Adenoviral cardiotrophin-1 transfer improves survival and early graft function after ischemia and reperfusion in rat small-for-size liver transplantation model

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Keywords
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Summary

This study was to investigate the effect of donor liver adenoviral cardiotrophin-1 (CT-1) gene transfer on early graft survival and function in rat small-for-size liver transplantation. We constructed a recombinant murine CT-1 adenoviral vector. Donor rats were transduced *in vivo* with adenoviruses expressing CT-1 (AdCT-1) or control vector (AdEGFP). Livers were harvested 4 days later, reduced to 40% of weight, and transplanted. A syngeneic rat orthotopic liver transplantation model was performed using 40% small-for-size grafts. Graft survival, liver function, hepatic architecture change, the degree of necrosis and apoptosis, and cell survival signaling pathways were assessed. AdCT-1 pretreatment markedly improved liver function and the survival of small-for-size grafts. In the CT-1 treatment group, hepatic architecture was well protected, apoptotic and necrotic cells were reduced; anti-apoptotic protein bcl-2 was up-regulated and pro-apoptotic cleaved caspase-3 was down-regulated, cell survival signaling pathways were activated by phosphorylation of protein kinase B (Akt), extracellular-regulated kinase (ERK) and Signal transducer and activator of transcription-3 (Stat-3) after transplantation. In conclusion, donor liver adenoviral CT-1 transfer ameliorated ischemia/reperfusion injury by decreasing hepatic necrosis and apoptosis in small-for-size liver transplantation, mediated in part by activation of the Akt, ERK, and Stat-3 survival signaling pathways. These results may provide a potential clinical strategy to improve the outcome of small-for-size liver grafts.

Introduction

Liver transplantation has been widely used as the therapy for patients with end-stage liver diseases. However, the shortage of donor organs limits the application of liver transplantation. In recent years, living donor liver transplantation (LDLT) has been a valuable alternative in solving the problem of organ shortage, and has shown significantly improved clinical outcomes [1]. However, with reduction of graft size, chances of primary graft nonfunction and complications also increase [2,3]. Small-for-size grafts encounter more risks of failure after reperfusion, including microcirculatory damage [4], more severe inflammatory responses [5], accelerated acute rejection processes [6], and impaired regeneration [7]. These effects frequently result in liver failure and associated coagulopathy, ascites, prolonged cholestasis, and encephalopathy. Collectively, this disorder is termed 'small-for-size syndrome'. However, effective therapeutic strategies to overcome this syndrome have not yet been thoroughly investigated in liver transplantation using small-for-size grafts.

Apart from the common ischemia/reperfusion injury (IRI), small-for-size grafts also suffer mechanical injury related to hemodynamic force. At the time of reperfusion, excessive blood inflow (in relation to the graft size)

generates more reactive oxygen species [8]. This, in turn, increases the susceptibility of liver cells to apoptotic stimuli and to mechanical injury associated with transient portal hypertension in small-for-size grafts. Recent studies have further demonstrated that early over-expression of cell death signals including apoptosis was instrumental in small-for-size graft damage [5,9]. Thus, therapeutic strategies targeting on cell survival/apoptosis pathways may be an effective approach to improve graft function in small-for-size liver transplantation.

Cardiotrophin-1 (CT-1) is a member of IL-6 family of cytokines that binds to a specific receptor that contains gp130 and the leukemia inhibitory factor receptor [10]. It was first identified by its ability to induce hypertrophy of cardiac myocytes [11] and was later shown to protect the heart from IRI [12] and support survival of developing motoneurons [13]. Recently, studies have shown that CT-1 is also expressed in both parenchymal and nonparenchymal liver cells and exerts potent anti-apoptotic effects on hepatocytes by activation of cell survival signaling pathways including signal transducer and activator of transcription-3 (Stat-3), extracellular-regulated kinase (ERK) 1/2 and protein kinase B (Akt) [14]. Recent studies further showed that CT-1 is an essential factor in the natural defense of the liver against apoptosis, CT-$1^{-/-}$ mice are more susceptible to Fas-induced apoptosis in the liver [15]. Administration of exogenous CT-1 to rat models of fulminant hepatic failure and mouse models of concanavalin-A-induced hepatitis protected the liver from injury by promoting cell repair and in terms of anti-apoptotic properties [14,16]. Furthermore, CT-1 significantly defended against liver warm IRI and mediated the protective effect of ischemic preconditioning [17]. Based on these data, we hypothesized that exogenous CT-1 may attenuate small-for-size graft injury, which presents with early over-expression of cell death signals after cold ischemia/warm reperfusion. Adenoviral-mediated gene delivery to the liver is highly effective even under conditions of cold organ storage [18–20]. Studies have demonstrated that over-expression of SOD or HO-1 in donor liver using recombinant adenovirus reduced IRI and improved survival after small-for-size liver transplantation [19,21]. Accordingly, the present study was designed to determine whether gene delivery of CT-1 could improve the outcome of small-for-size liver transplantation and the possible mechanisms underlying the same.

Materials and methods

Construction of plasmids and preparation of adenoviral vectors

Murine cDNA encoding CT-1 gene was cloned across the BamH I–Sal I sites of plasmid PGEX4T-3 and then into the pAdTrack-CMV shuttle vector via KpnI–XbaII sites. The pAdTrack-CMV vector encodes EGFP under the control of a separate cytomegalovirus (CMV) promoter to act as a marker for transfection. The plasmid was linearized by restriction digestion with PmeI and cotransformed into E. coli BJ5183 cells with pAdEasy-1. Recombinants were selected for kanamycin resistance and confirmed by restriction analysis. The linearized recombinant plasmid was transfected into an adenovirus packaging cell line HEK293, using Lipofectamine in T-25 flasks according to the manufacturer's instructions. Transfection and viral productions were monitored by GFP expression. For viral purification, cells were harvested and subjected to four freeze/thaw cycles in dry ice/methanol and centrifuged to obtain a crude viral stock. The crude viral stock was further amplified and purified by CsCl banding. The concentrations of AdCT-1 and AdEGFP were determined by plaque-forming assay, and expressed as plaque-forming units (pfu). Both vectors were diluted to 3×10^9 pfu/ml with 1 ml saline for intravenous injection to donor rats.

Animals

Syngeneic male Lewis rats (8–12 weeks of age, body weights 250–320 g; Vital River Experimental Animal Co, Beijing, China) were used as donors and recipients to exclude immunologic interference. The rats were kept under constant environmental conditions with a 12 h light–dark cycle and free access to food and water. The rats were fasted 12 h before the operation. All animal experiments were conducted in accordance with the guidelines approved by the Chinese Association of Laboratory Animal Care.

Experimental design and surgical procedure

The experiments were conducted in three groups of rats: (i) AdCT-1 group, (ii) AdEGFP control group, and (iii) saline control group. AdCT-1, AdEGFP or 1 ml 0.9% saline was injected via the penile vein into prospective donor animals. Four days later, the donor liver was harvested. A rat nonarterialized orthotopic liver transplantation model without veno-venous bypass was performed according to the techniques previously described by Kamada [22], with slight modifications. The lobe ligation technique was used to reduce the graft size on the back table. The median lobe of the liver was selected to be the graft and the median ratio of the graft weight-to-recipient liver weight was 39.6% (range: 36–45%). Liver grafts were stored in 4 °C saline with a preservation time of 80 min for all experimental groups. Donors and recipients were anesthetized with ether following an intraperitoneal injection of atropine (0.05 mg/kg), and blood and liver tissue

samples were obtained at 2, 6, and 24 h (n = 6/time point/group) after reperfusion of recipients.

Detection of CT-1 and EGFP expression in the post-transplanted liver

To evaluate the effectiveness of donor liver CT-1 gene transfer in the post-transplanted liver, western blotting assay was performed to examine the protein expression of CT-1 at 6 and 24 h in the post-transplanted liver. Furthermore, using enhanced green fluorescent protein (EGFP) gene as a marker, we evaluated the transfer efficiency of CT-1 in the post-transplanted livers indirectly by observing the green fluorescence of liver frozen section.

Survival study

Fifteen rats in the Saline/AdEGFP control and AdCT-1 treatment groups with 40% liver transplantations were used for the survival study. Rats that lived for more than 7 days after transplantation were considered survivors.

Biochemical examination

Blood samples collected from the recipients after reperfusion were immediately centrifuged to obtain serum. The serum samples were stored at −80 °C for subsequent analysis. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard automatic Analyzer (Hitachi 7600-10; Hitachi High-Technologies Corporation, Tokyo, Japan).

Morphological examination by light and electron microscopy

Liver tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections (4-μm) were stained with hematoxylin and eosin. Ten random fields of each H–E stained section were assessed for necrosis by standard morphologic criteria (e.g. loss of architecture, vacuolization, karyolysis, and increased eosinophilia), and the percentage of necrotic area was quantified using NIH image analysis software. All histological evaluations were done in a double blinded fashion.

For electron microscopic analysis, liver tissues were fixed in 2.5% dialdehyde for 2 h, postfixed in osmium tetroxide, stained *en bloc* in aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon-812 epoxy resin; ultra thin sections were stained with aqueous uranyl acetate and Reynolds' lead citrate, and examined using a JEM-1200 electron microscope (Jeol Ltd, Tokyo, Japan).

Apoptotic cell detection by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

The paraffin sections of liver biopsies obtained at 6 and 24 h were examined for apoptotic cells using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to the instructions of the commercial kit (Apop Tag, Intergen Co., NY, USA). Ten random fields were counted for each TUNEL-stained tissue sample in a blinded matter. Classic TUNEL positivity was characterized by focal nuclear staining and the presence of intact nuclear and cell membrane integrity in apoptotic cells. Three different liver sections from different lobules were analyzed.

Western blots for CT-1, the cell survival pathways (Akt, ERK, and Stat-3), bcl-2 and cleaved caspase-3

Liver tissues were homogenized and proteins were extracted using a commercial extraction kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations were then determined by the bicinchoninic acid (BCA) protein assay kit (Shenergy Biocolor, Shanghai, China). Equal amounts of the protein samples (50 μg/sample) were separated by 10–15% SDS-PAGE, followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes (Roche, Indianapolis, IN, USA). The membranes were then incubated with the primary antibodies: β-actin, α-tubulin, CT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt, phospho-Akt^{ser473}, P42/P44ERK, phospho-p42/P44ERK, Stat-3, phospho-Stat-3^{Tyr705}, cleaved caspase-3, and bcl-2 (Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C; followed by a anti-goat or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The immunoreactive signals were visualized using enhanced chemiluminescence detection reagents (Pierce Biotechnology). Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY, USA).

Statistical analysis

Results were expressed as mean ± SEM. Comparisons between the groups at different time-points were analysed using the Student's t-test or ANOVA where appropriate. The log-rank test was used to test the equality of the three survival rates. A *post hoc* multiple comparison of survival rates between the three groups was conducted with a log-rank test, followed by a Bonferroni correction. Statistical analyses were conducted using 13.0 SPSS computer software (SPSS Inc., Chicago, IL, USA) and statistical significance was inferred at $P < 0.05$.

Results

Donor liver CT-1 gene transfer leads to CT-1 over-expression in post-transplanted grafts

Western analysis showed that adenoviral gene transfer of CT-1 resulted in a significant increase in CT-1 protein levels at 6 and 24 h after reperfusion in small-for-size grafts (Fig. 1a). These findings were confirmed by the presence of EGFP in the post-transplanted liver at 6 h in the AdEGFP and AdCT-1 groups (Fig. 1b). No differences in CT-1 expression were observed between the saline and AdEGFP transplanted graft livers. No EGFP expression was noted in the saline control group.

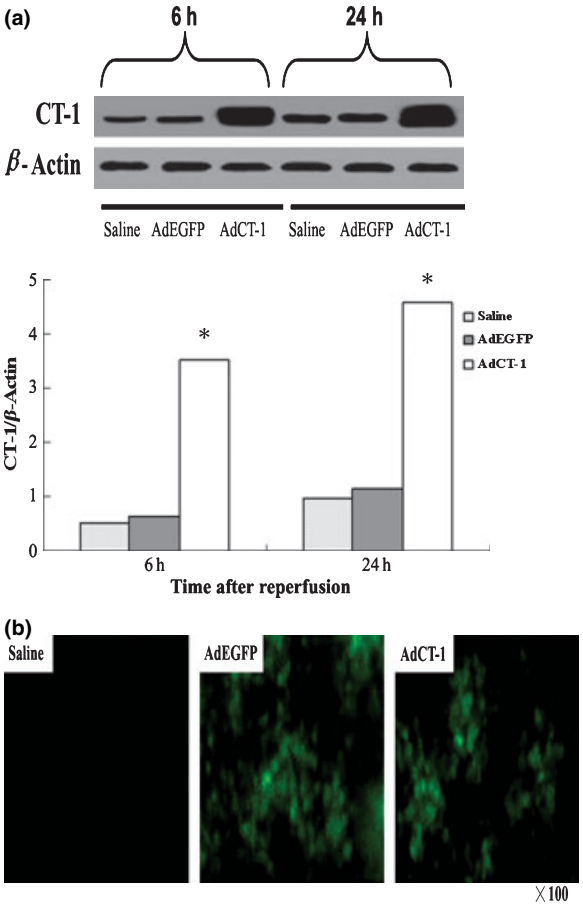


Figure 1 (a) Representative Western blot demonstrated a marked increase of CT-1 protein expression at 6 and 24 h in the AdCT-1 pre-treatment group, when compared to controls. No significant differences of CT-1 expression were observed between the saline and AdEGFP control group. *$P < 0.01$ versus saline and AdEGFP control groups. Each immunoblot represents three independent experiments. (b) Enhanced green fluorescence protein (EGFP) expression was determined by fluorescent microscopy at 6 h after reperfusion. GFP-positive cells were detected in the AdCT-1 treatment and AdEGFP control groups; no EGFP gene expression was observed in the saline control group (Original magnification, ×100).

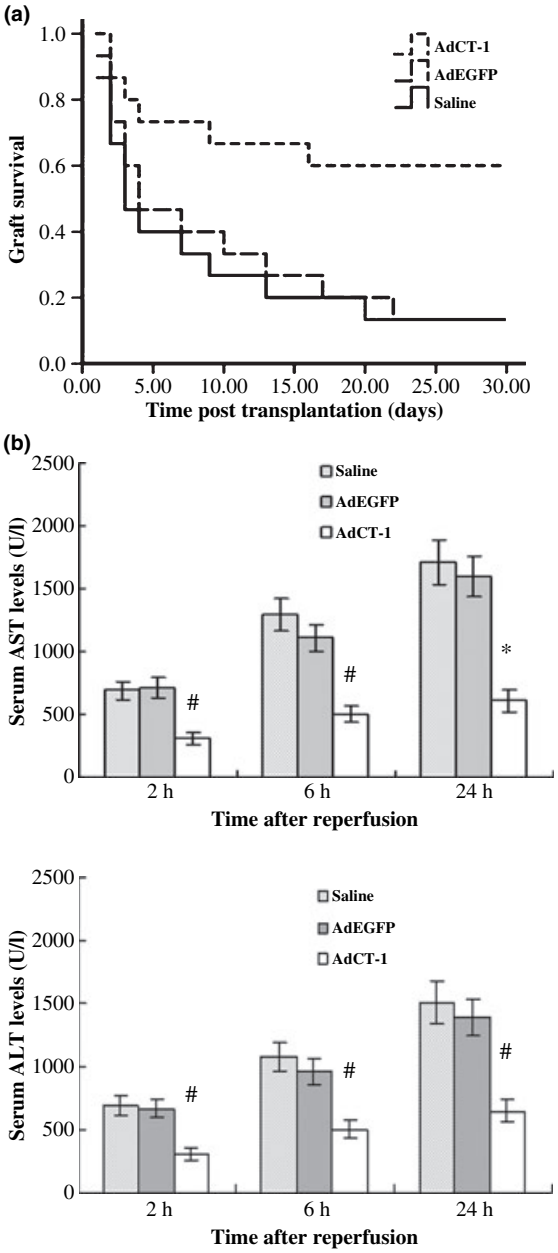
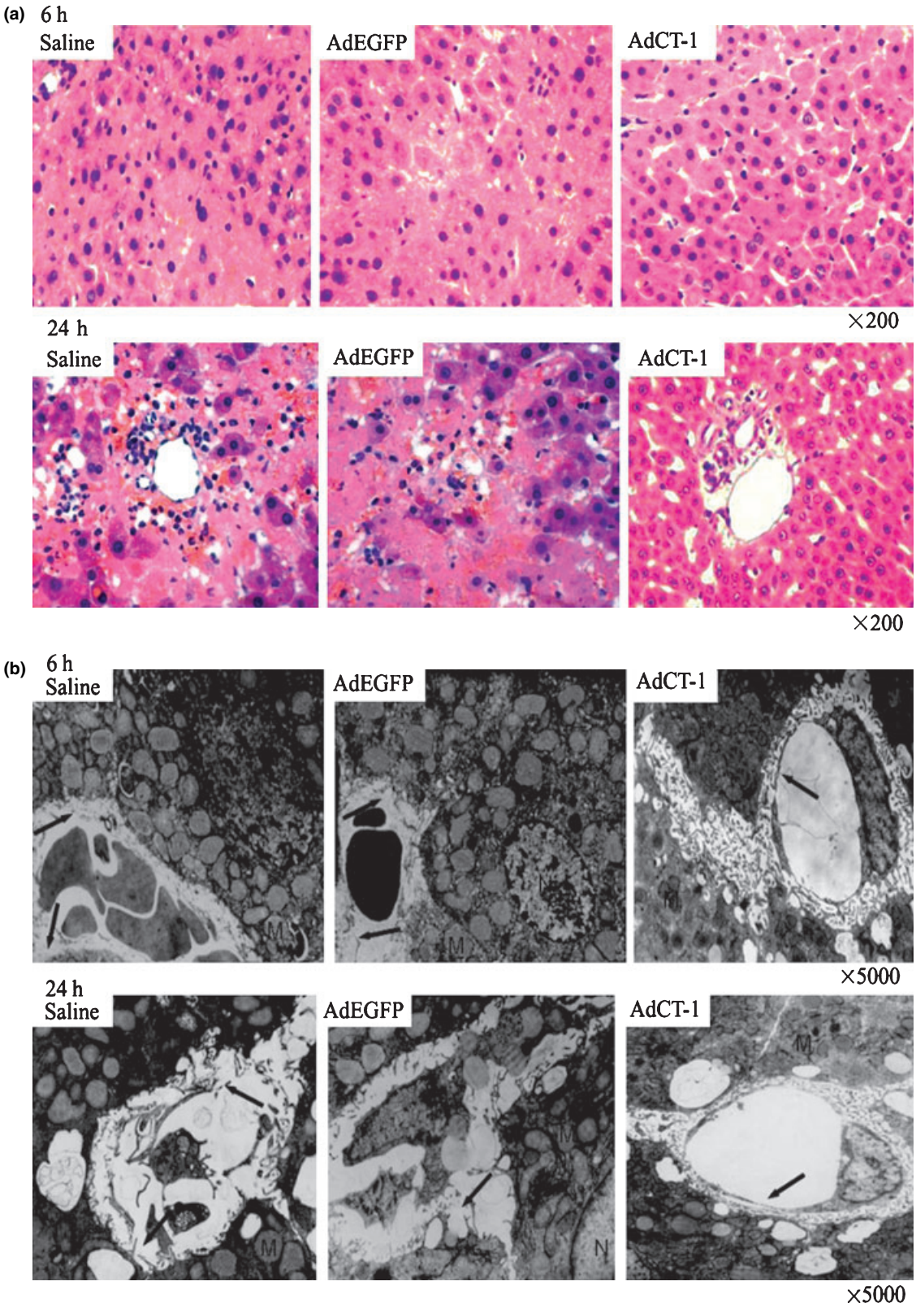


Figure 2 (a) Kaplan–Meier plot showing the pattern of survival of recipients after small-for-size liver transplants (n = 15/group). In the AdCT-1 treatment group, survival rates were significantly improved compared with the two control groups. (b) The serum AST and ALT levels at 2, 6, and 24 h after reperfusion were significantly lower in the AdCT-1 group than in the saline and AdEGFP groups; no differences were observed between the two control groups. #$P < 0.05$, *$P < 0.01$ versus saline and AdEGFP control groups. AST, aspartate aminotransferase; ALT, alanine aminotransferase.



CT-1 gene transfer improved animal survival and liver graft function

A Kaplan–Meier Curve was used to describe the survival process. In general, the differences in survival rates between the three groups were significant, and the survival rate of the AdCT-1 group was improved compared to the AdEGFP and saline control groups. The 7-day survival rate was significantly improved from 33.3% (5/15) in the saline control group and 40% (6/15) in AdEGFP control group to 73.3% (11/15) in the AdCT-1 group ($P < 0.05$; Fig. 2a). Plasma levels of AST and ALT were significantly lower in the AdCT-1 group compared to the saline and AdEGFP control groups at 2, 6, and 24 h after reperfusion (Fig. 2b).

CT-1 gene transfer preserved the liver architecture of small-for-size grafts

Demonstrated by hematoxylin–eosin staining, apparent hepatocellular degeneration accompanied by scattered necrotic and apoptotic cells were observed in the AdEGFP and saline control groups at 6 h after reperfusion. Severe disruptions of lobular architecture and more severe hepatocellular degeneration and apoptosis with extensive necrosis were subsequently noted at 24 h after reperfusion. In contrast, hepatic lobular architecture was well preserved in the AdCT-1 group at 6 and 24 h after reperfusion (Fig. 3a).

The hepatic ultrastructural changes were then examined by electron microscopy. In the saline and AdEGFP control groups, mitochondria swelling and degeneration of cytoplasm of hepatocytes were observed at 6 h after reperfusion, accompanied by an irregular large gap of sinusoidal endothelial cells and loss of microvilli in the space of Disse. Twenty-four hours after reperfusion, the integrity of endothelial cells was disrupted. In contrast, the AdCT-1 treatment group contained hepatocytes and sinusoidal lining cells with a normal appearance (e.g. intact sinusoidal lining cells, elliptical mitochondria with well-visualized cristae, and abundant microvilli in the space of Disse) at 6 and 24 h after reperfusion (Fig. 3b).

CT-1 gene transfer attenuated hepatic necrosis and apoptosis after transplantation of small-for-size grafts

To further assess the protective effect of CT-1 on IRI, we quantified the necrotic and apoptotic cells in small-for-size grafts. The percentage area of necrosis was quantified on H–E stained sections as described in the methods section. Hepatic necrosis developed mainly in the periportal and midzonal regions of the liver lobule (Fig. 3a). The area of necrosis was lower in the AdCT-1 group compared to the saline and AdEGFP groups (6 h: 3.3 ± 1.1 vs. 13.3 ± 2.3 and 12.6 ± 2.1, respectively; 24 h: 6.4 ± 1.3 vs. 22.9 ± 3.5 and 20.8 ± 2.8, respectively; $P < 0.01$).

To examine the level of apoptotic cells, TUNEL labeling was performed in small-for-size liver grafts. Some apoptotic nuclei were noted at 6 h and a significant increase occurred at 24 h after reperfusion. The frequency of positive apoptotic cells was markedly lower in the AdCT-1 treatment group at 6 and 24 h, when compared with control groups (Fig. 4a and b). To evaluate whether CT-1 therapy affected apoptotic networks, we examined the expression of anti-apoptotic protein bcl-2 and pro-apoptotic cleaved caspase-3 protein contents by Western analysis. As shown in Fig. 5, significantly increased bcl-2 protein contents and decreased cleaved-caspase-3 were noted in the AdCT-1 group compared to the two control groups.

CT-1 gene transfer activated cell survival signaling pathways by Akt, ERK and Stat-3 phosphorylation in small-for-size grafts

To investigate the possible molecular mechanism of CT-1-mediated protection of small-for-size grafts, the Akt, ERK, and Stat-3 survival pathways were examined in small-for-size grafts via western blotting. Results demonstrated that there were no significant differences in total Akt, ERK, and Stat-3 protein contents between the control and treatment groups (Fig. 6). AdCT-1 pretreatment induced a significant increase in phospho-Akt, phospho-ERK, and phospho-Stat-3 protein contents at 6 and 24 h after reperfusion in small-for-size grafts.

Figure 3 In the saline and AdEGFP groups, H–E staining showed apparent hepatocellular degeneration accompanied by scattered necrotic and apoptotic cells at 6 h after reperfusion; severe disruption of lobular architecture, more severe hepatocellular degeneration and apoptosis with extensive necrosis were present at 24 h after reperfusion. Whereas minimal damage was observed in the AdCT-1 treatment group (a; original magnification, ×200). Likewise, in the saline and AdEGFP groups, hepatic ultrastructural changes noted by electron microscopy included mitochondria swelling accompanied by an irregular large gap of sinusoidal endothelial cells, loss of microvilli at 6 h after reperfusion, and disruption of the endothelial cell integrity at 24 h after reperfusion. While in AdCT-1 group, both the cell nucleus and cellular organelles have no significant breakdown. M, mitochondrial; N, nucleus; arrow, sinusoidal lining cells (b; original magnification, ×5000).

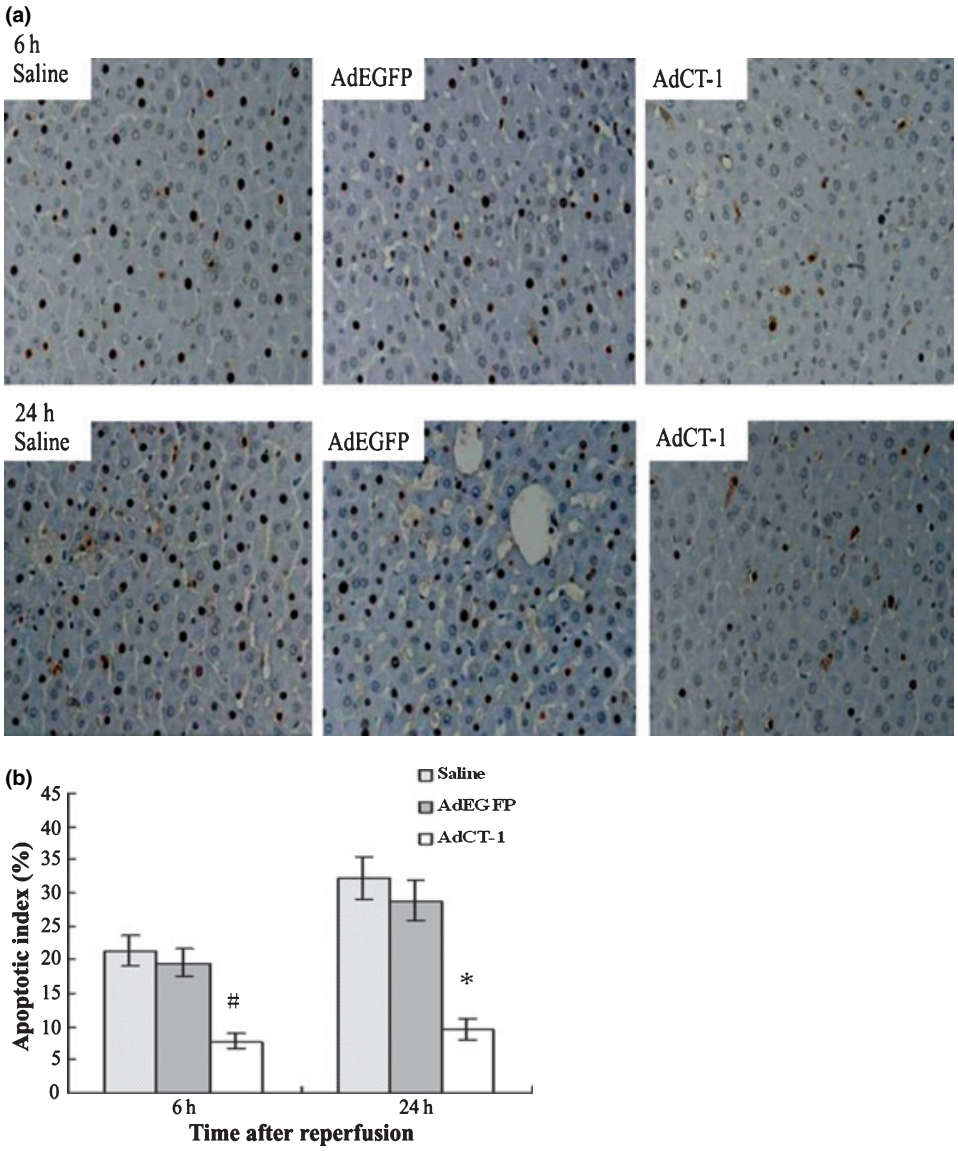


Figure 4 (a) Representative graph of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining at 6 and 24 h after reperfusion. The frequency of apoptotic cells (defined as dark brown spots) was diminished in the AdCT-1 group compared with the saline and AdEGFP groups (Original magnification, ×400). (b) Graph of the average percentage of TUNEL-positive cells at 6 and 24 h in small-for-size grafts. The apoptotic index, calculated as the percentage of TUNEL-positive nuclei divided by the counter- stained nuclei, was significantly diminished in the AdCT-1 group (*$P < 0.01$, compared with saline and AdEGFP control groups).

Discussion

To rescue the small-for-size grafts from acute-phase injury after liver transplantation, therapeutic strategies for attenuation of small-for-size graft injuries have been investigated according to its distinct pattern of hepatic IRI in clinical transplantation and animal experiments [23–28]. Recent studies in our laboratory have also revealed that adenosine A_{2A} receptor activation, biliverdin and mesenchymal stem cell over-expression of hepatocyte growth factor conferred protection against small-for-size graft injury after liver transplantation [29–31]. The present studies on the protective effect of CT-1 transduction in liver donors on IRI in small-for-size grafts have demonstrated that CT-1 over-expression: (i) improved liver function and preserved hepatocyte architecture with resultant prolongation of graft survival; (ii) was associated with decreased hepatic necrosis and apoptosis; and (iii) activated cell survival signaling pathways including Akt, ERK and Stat-3 in transplanted livers.

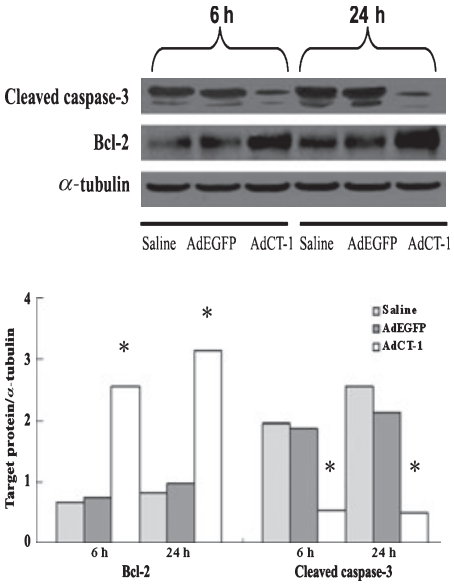


Figure 5 Anti-apoptotic bcl-2 levels were significantly up-regulated and pro-apoptotic cleaved caspase-3 was markedly down-regulated in the AdCT-1 group compared to the saline and AdEGFP groups. (*P < 0.01 compared with saline and AdEGFP control groups).

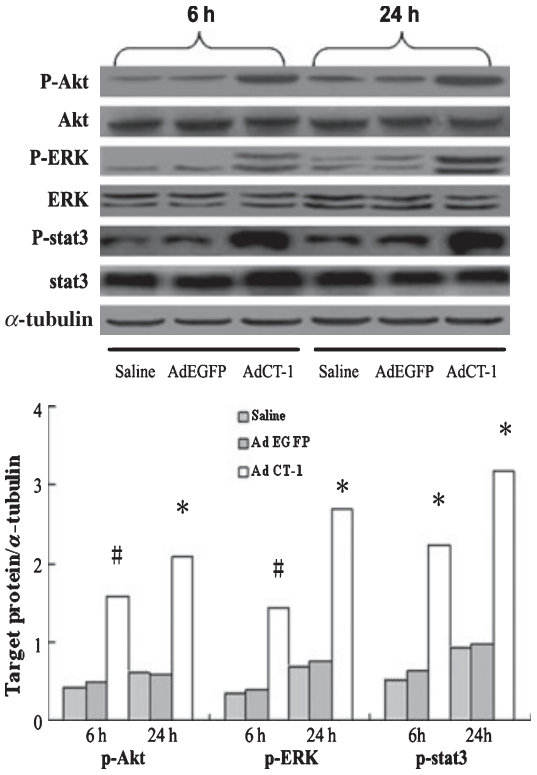


Figure 6 No significant differences in total Akt, extracellular-regulated kinase (ERK) and Signal transducer and activator of transcription-3 (Stat-3) were observed between the three groups. However, p-Akt, p-ERK and p-Stat-3 levels were significantly up-regulated in the AdCT-1 group compared to the saline and AdEGFP groups. #P < 0.05, *P < 0.01 versus saline and AdEGFP control groups. Each immunoblot represents three independent experiments.

Cardiotrophin-1 is a member of IL-6 family of cytokines, and has been shown to display potent cytoprotective effects on hepatocytes [14–16]. Recent research demonstrated that CT-1 could protect the liver against a variety of damaging insults [14–17]. However, the effect of CT-1 on liver transplantation using small-for-size grafts has not yet been studied. In the present study, we demonstrated that AdCT-1 pretreatment preserved hepatic architecture and improved the survival of small-for-size grafts. To the best of our knowledge, this is the first report on the efficacy of CT-1, over-expressed in donor livers via adenoviral gene transfer, in the preservation of small-for-size graft function.

Hepatic cold IRI induces cell death through apoptosis and necrosis [32] or a combination of both called necrapoptosis [33]. Although necrosis is a major feature of liver damage after IRI, increasing evidence also points to apoptosis as a critical mechanism during the initial phase of hepatic cold ischemia/warm reperfusion injury in liver transplantation [34,35]. Therapeutic strategies counteracting apoptosis in liver grafts with caspase inhibitor [36,37] or by adenoviral bcl-2 gene transfer have already shown beneficial effects on liver transplantation [20]. Several studies have shown that apoptosis is a critical process involved in early small-for-size graft damage [5,9,29–31]. In the current study, CT-1 pretreatment decreased the level of hepatic necrosis compared to the controls groups at 6 and 24 h after reperfusion. In addition, H–E staining showed some evidence of apoptotic cells at 6 h, which became prominent at 24 h after reperfusion, and the frequency of TUNEL-positive cells in AdCT-1 group was lower compared to controls. Western analysis also showed that AdCT-1 pretreatment promoted the expression of Bcl-2 and prevented the activation of the caspase-3. Caspase-3 activation is the key step in the apoptosis; it is most uniquely associated with apoptosis [32]. Bcl-2 prevents the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor from mitochondria into the cytosol [38]. Although TUNEL positive staining might have partially resulted from necrotic cells, in combination with other data including cell morphological changes and cleaved caspase-3 examination, we thought that TUNEL assay was a consequence, at least in part, of apoptosis in present study. Overall, our data suggest that cold I/R causes hepatocyte death through necrosis and apoptosis in small-for-size grafts and CT-1 decreases liver damage by attenuating both pathways.

The dynamic balance of cell survival and death is critical in determining the fate of hepatocytes subsequent to

reperfusion injury. To obtain further insight into the possible molecular mechanism of the protective effect of CT-1 on IRI in small-for-size liver grafts, we examined the effect of CT-1 on cell survival pathways after reperfusion. Our results showed that p-Akt, p-ERK, and p-Stat-3 protein contents were higher in the AdCT-1 treatment group than the control groups, which may be reflective of increased activation of survival signaling pathways.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is an important survival pathway for cell growth and proliferation [39]. Akt activation has been shown to be an important regulator of cell survival and apoptosis [40], and stimulates downstream phosphorylation of endothelial nitric oxide synthase (eNOS) resulting in NO synthesis [41]. Previous studies have suggested that endogenous NO derived from eNOS plays an important role in the attenuation of sinusoidal constriction during the early phase of small-for-size grafts [3]. Moreover, FK409 (a potent NO releaser) has been shown to ameliorate small-for-size liver graft injury by attenuation of portal hypertension and down-regulation of the Egr-1 pathway [42]. Recently, some investigators have demonstrated the beneficial impact of Akt activation on liver dysfunction during liver warm or cold IRI [43,44]. In the present study, Donor CT-1 gene transfer activated Akt phosphorylation in post-transplanted livers. Based on these findings, we speculate that Akt-activated endogenous NO synthesis may play a protective role in small-for-size graft injury. However, further studies are required to investigate whether exogenous CT-1 could increase eNOS expression in rat small liver grafts.

Extracellular-regulated kinase, one of MAPK signaling pathways, plays a critical anti-apoptotic role in the regulation of cell growth and differentiation [45]. It is distinctly different from the other two aggravating signal pathways (p54/p46 JNK and p38MAPK). Recent research showed that ERK pathway activation may play a protective effect on cell growth and differentiation after small-for-size liver transplantation [46]. CT-1 has been shown to activate the ERK pathway in different cell types and organ systems including the liver [14]. In the present study, ERK phosphorylation was also increased after reperfusion in the AdCT-1 pretreatment group.

Signal transducer and activator of transcription-3 is an important transcription factor in hepatic parenchymal and nonparenchymal cells, which constitutes an essential component of survival pathways that defend hepatocytes against Fas-mediated liver injury [47]. Recent research showed that STAT3-C transduced into liver grafts by adenoviral vectors not only protected against IRI, but also accelerated liver regeneration in a rat partial liver transplantation model [48]. Study by Marques has confirmed that CT-1 is an indispensable factor for Stat-3 activation in the Fas-induced liver damage [15]. Additional studies have also revealed that CT-1 is a critical mediator of Stat-3 activation in rat livers exposed to warm IRI [17]. Consistent with previous studies, we also observed marked activation of Stat-3 in graft livers after reperfusion in the AdCT-1 pretreatment group. Therefore, we hypothesize that Stat-3 activation may be one of the key protective mechanisms of CT-1 in small-for-size graft injury.

The mechanisms of small-for-size graft failure have been studied in recent years [5–9,26,49]. Severe inflammatory response triggered by shear stress and impaired regeneration also play important roles in small-for-size graft failure after liver transplantation. The present study examined the protective potential of CT-1 on small-for-size graft injury, which is at least in part mediated by activation of the Akt, ERK, and Stat-3 survival pathways. Previous studies have shown that CT-1 also has anti-inflammatory [50,51] and liver repair properties [16,52]. Future research in our laboratory will investigate whether CT-1 can promote liver regeneration and attenuate the inflammatory status of small-for-size grafts.

Gene therapy has been an area of intensive research in the field of organ transplantation in recent years. Potential applications of gene transfer technology include prevention of graft rejection and amelioration of cold IRI [53,54]. Effective gene therapy requires a reliable method of gene transfer into the target organ to produce the required levels of functional protein. Adenoviral vectors have a high affinity for liver tissue and DNA is not incorporated into the genome, they represent appropriate vectors for transient hepatic gene expression. To date, effective methods of gene transfer include *ex vivo* gene transfer during preservation or donor pretreatment with Ad vector by intravenous infusion [18,48]. Studies demonstrated that donor pretreatment provides very early transgene expression and is well suited for gene delivery in the modulation of preservation injury or early IRI [18,54].

Consistent with previous reports, our finding has shown that a single intravenous infusion of AdCT-1 into donor livers 4 days before harvest resulted in exogenous CT-1 expression in transplanted livers. In a clinical setting, application of gene transfer after diagnosis of brain death is not practical. However, in the context of LDLT, application of gene transfer to donors is potentially possible. Donor liver CT-1 transfer not only decreased graft injury associated with graft harvest, storage and reperfusion in the recipient, but also minimized the risk of liver failure in the donor.

In conclusion, the data generated in this study support the hypothesis that over-expression of CT-1 by adenoviral gene transfer improves survival and graft function by reducing necrosis and apoptosis in rat small-for-size liver

grafts. The underlying mechanism of this protective effect may at least involve the activation of the Akt, ERK and Stat-3 survival signaling pathways in postischemic liver issue. Although the precise mechanisms require further clarification, the present study provides the rationale for a novel therapeutic approach using AdCT-1 gene transfer to maximize the availability of small-for-size liver grafts.

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Authorship

JS: design research project, perform experiments, write paper, collect and analyze data. Y-WZ: perform experiments. A-HY: establish animal model and perform experiments. YY: perform experiments. Z-YH: establish animal model. L-YP: establish animal model and collect data. G-QL: collect data. X-CL: analyze data. FZ: perform experiments. G-QS: perform experiments. X-HW: design research project and analyze data.

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